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GENETIC TRANSFORMATION AND HYBRIDIZATION

Increased α-tocopherol content in soybean seed overexpressing the *Perilla frutescens γ-tocopherol methyltransferase* gene

Venkata S. Tavva · Yul-Ho Kim · Isabelle A. Kagan · Randy D. Dinkins · Kyung-Hwan Kim · Glenn B. Collins

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Abstract Tocopherols, with antioxidant properties, are synthesized by photosynthetic organisms and play important roles in human and animal nutrition. In soybean, γ-tocopherol, the biosynthetic precursor to α-tocopherol, is the predominant form found in the seed, whereas α-tocopherol is the most bioactive component. This suggests that the final step of the α-tocopherol biosynthetic pathway catalyzed by γ-tocopherol methyltransferase (γ-TMT) is limiting in soybean seed. Soybean oil is the major edible vegetable oil consumed, so manipulating the tocopherol biosynthetic pathway in soybean seed to convert tocopherols into more active α-tocopherol form could have significant health benefits. In order to increase the soybean seed α-tocopherol content, the *γ-TMT* gene isolated from *Perilla frutescens* was overexpressed in soybean using a seed-specific promoter. One transgenic plant was recovered and the progeny was analyzed for two generations. Our results demonstrated that the seed-specific expression of the *P. frutescens γ-TMT* gene resulted in a 10.4-fold increase in the α-tocopherol content and a 14.9-fold increase in the β-tocopherol content in T2 seed. Given the relative contributions of different tocopherols to vitamin E activity, the activity in T2 seed was calculated to be 4.8-fold higher than in wild-type seed. In addition, the data obtained on lipid peroxidation indicates that α-tocopherol may have a role in preventing oxidative damage to lipid components during seed storage and seed germination. The increase in the α-tocopherol content in the soybean seed could have a potential to significantly increase the dietary intake of vitamin E.

Keywords HPLC · Lipid peroxidation · *Perilla frutescens* · Soybean · α-Tocopherol · γ-Tocopherol methyltransferase · Transgenic plants · Vitamin E

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V. S. Tavva · G. B. Collins (✉)
Department of Plant and Soil Sciences,
University of Kentucky,
1405 Veterans Road, Lexington, KY 40546-0312, USA
e-mail: gcollins@uky.edu

Y.-H. Kim
Crop Environment and Biotechnology Division, National Institute of Crop Science,
209 Seodun-Dong, Suwon 441-857, South Korea

I. A. Kagan · R. D. Dinkins
USDA-ARS Forage-Animal Production Research Unit,
1100 Nicholasville Road, Lexington, KY 40546-0091, USA

K.-H. Kim
National Institute of Agricultural Biotechnology, Rural Development Administration,
Suwon 441-707, South Korea

Introduction

Cellular and molecular biology approaches to manipulating vitamin E composition in plants, with the aim of improving the α-tocopherol content in the human diet, have been a major focus of research in recent years (Cahoon et al. 2003; Collakova and DellaPenna 2003; Grusak and DellaPenna 1999; Kim et al. 2005; Van Eenennaam et al. 2003). Vitamin E collectively represents a group of structurally related compounds, α-, β-, γ- and δ-tocopherols, and four corresponding unsaturated derivatives, α-, β-, γ- and δ-tocotrienols. All tocopherols are amphipathic molecules consisting of a polar chromanol head group derived from homogentisic acid and a lipophilic tail derived from phytyl-diphosphate (Bramley et al. 2000; Savidge et al. 2002). Tocotrienols differ from tocopherols only in having a lipophilic tail derived from geranylgeranyl diphosphate with double bonds at carbon

positions 3′, 7′ and 11′ (Cahoon et al. 2003; Munne-Bosch and Alegre 2002). The biological activities of α-, β-, γ- and δ-tocopherols vary with the number and position of methyl groups on the chromanol ring (Fig. 5a) (Bramley et al. 2000; Fryer 1992), and vitamin E activities of α-, β-, γ- and δ-tocopherols are 100, 50, 10 and 3% equivalent to that of α-tocopherol activity (Kamal-Eldin and Appelqvist 1996; Sheppard et al. 1993; Shintani and DellaPenna 1998).

Tocopherols are synthesized exclusively by photosynthetic organisms. Usually, α-tocopherol is the predominant form found in leaves, while γ-tocopherol and tocotrienols accumulate to higher levels in seed of many plant species (Demurin et al. 1996; Tan 1989). The aromatic ring of tocopherol interacts with reactive oxygen species (Fryer 1992), and for this reason, tocopherol is believed to be critical to protect fatty acids in membranes from oxidative degradation (McKersie et al. 1990). Furthermore, it has been suggested that tocopherol is required to scavenge reactive oxygen species produced during the process of photosynthesis (Fryer 1992). Although all tocopherols and tocotrienols are potent antioxidants in vitro, α-tocopherol is most active in terms of vitamin E activity, partly because it is retained in the human body preferentially to other tocopherols and tocotrienols (Traber and Sies 1996). Many studies have demonstrated that the antioxidant properties of α-tocopherol are strongly implicated in various aspects of human health, including heart disease, cancer and inflammatory responses (Brigelius-Flohe and Traber 1999; Rimm et al. 1993; Stampfer et al. 1993). For this reason, there is an interest in developing ways to increase the intake of natural tocopherols in human and animal diets.

Twenty to thirty percent of vitamin E in human diet in the United States comes from oil-based products such as margarines, dressings and mayonnaise (Eitenmiller 1997; Sheppard et al. 1993). Soybean oil accounts for ∼70% of the edible oil consumed by humans in the United States and 30% of the worldwide oil consumption (USDA Foreign Agriculture Service 2003). In oil seed, the major tocopherols are the highly abundant γ-tocopherol (60–65% of the total) and δ-tocopherol (20–26% of the total) (Tan 1989). Since the vitamin E activity of γ-tocopherol is only about one-tenth of that of α-tocopherol, the relative value of diets with higher levels of γ-tocopherol is low. Furthermore, it has been known that α-tocopherol is the only type of vitamin E that human blood can maintain and transfer to cells when needed (Drevon 1991; Hosomi et al. 1997; Traber and Sies 1996). Obtaining therapeutic levels of vitamin E from the average diet is difficult without consuming large quantities of foods enriched in vitamin E (Shintani and DellaPenna 1998). Therefore, manipulating the seed tocopherol biosynthetic pathway in soybean to convert the less active tocopherols to the most bioactive α-tocopherol could have significant human health benefits and make this crop an attractive target for the improvement of tocopherol composition (Van Eenennaam et al. 2003). γ-Tocopherol methyltransferase (γ-TMT), the final enzyme in the tocopherol pathway, uses *S*-adenosylmethionine (SAM) to generate α- and β-tocopherols from γ- and δ-tocopherols, respectively (d'Harlingue and Camara 1985; Hofius and Sonnewald 2003; Koch et al. 2003). Consequently, seed-specific overexpression of the *γ-TMT* gene could be a way to convert the large food pool of γ-tocopherol into α-tocopherol in soybean seed. Increases in α-tocopherol content in soybean seed have previously been demonstrated using the *γ-TMT* gene from the model plant *Arabidopsis thaliana* (*At-VTE4*) (Kim et al. 2005; Van Eenennaam et al. 2003), and it has also been used to increase the α-tocopherol content in lettuce leaves (Cho et al. 2005).

The objective of this study was to increase the α-tocopherol content in soybean through seed-specific overexpression of the *γ-TMT* gene of *Perilla frutescens*, commonly known as the beefsteak mint plant, a native to East Asia. *P. frutescens* was chosen since it is also used as an oilseed crop, similar to soybean, and should express in soybean without the potential silencing problems that would be associated with overexpressing the endogenous soybean gene. In this work, we have shown that seed-specific overexpression of the *P. frutescens γ-TMT* (*PfTMT*) gene in soybean resulted in a significant increase in the seed α- and β-tocopherol content.

Materials and methods

DNA manipulations

The full-length *γ-TMT* gene (GenBank accession number AF213481) was amplified from cDNA prepared from developing seed of *Perilla frutescens* cv. Okdong (K.-H. Kim, unpublished data). The *PfTMT* gene was placed under a seed-specific promoter, vicilin (GenBank accession number X14076) and the poly A sequence from the *Agrobacterium tumefaciens* octopine synthase gene (GenBank accession number AF242881). The entire cassette (Vicilin P:*PfTMT*:OCS T) was cloned into the pCAMBIA1304 binary vector (CAMBIA, Canberra, Australia) for plant transformation, and the resultant construct was designated as p1304*PfTMT*. The T-DNA region of the p1304*PfTMT* binary vector contains a CaMV35S promoter-driven hygromycin gene expression cassette, a CaMV 35S promoter-driven GFP–GUS fusion gene expression cassette, and the Vicilin P:*PfTMT*:OCS T expression cassette (Fig. 1).

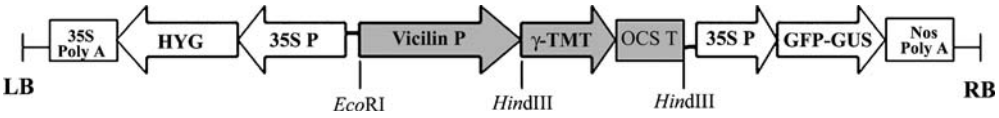

Fig. 1 Schematic representation of the binary vector constructed for soybean transformation. The T-DNA region of the p1304*PfTMT* binary vector showing the assembly of γ-TMT expression cassette (Vicilin P:γ-TMT:OCS T), hygromycin expression cassette (35S P:HYG:35S poly A) and GFP–GUS reporter fusion gene expression cassette (35S P:GFP–GUS:Nos poly A). γ-TMT: γ-tocopherol methyltransferase; OCS T: *Agrobacterium tumefaciens* octopine synthase poly A; 35S P: CaMV 35S promoter; 35S poly A: CaMV 35S poly A; HYG: hygromycin; GFP–GUS: green fluorescent protein – β-glucuronidase fusion gene; Nos poly A: *Agrobacterium tumefaciens* nopaline synthase poly A

Plant tissue culture and transformation

Somatic embryogenic cultures were initiated by plating immature (<6 mm) cotyledons (cv. Jack) on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 40 mg l^{-1} 2,4-D, 20 g l^{-1} sucrose, and 2 g l^{-1} phytagel (pH 7.0), and maintained in a culture room (23:1 h light/dark at 23°C) as detailed by Trick et al. (1997). Embryos were transferred to proliferation medium (MS-based medium containing 20 mg l^{-1} 2,4-D, 20 g l^{-1} sucrose, and 2 g l^{-1} phytagel, pH 5.8) for embryogenic proliferation of somatic embryos, with transfers made to fresh medium every 30 days. Green embryos from raspberry-shaped clumps were selected for transformation using a dissecting microscope (Zeiss SV6; Carl Zeiss, Oberkochen, Germany). Selected material was placed in the center of a Petri plate with proliferation medium for 4 days (approximately 100–150 mg of somatic embryos per plate). Transformation was carried out via particle bombardment with a gene gun (Dupont PDS1000; Bio-Rad Laboratories, Hercules, CA) using gold/DNA microprojectile preparations. Immediately after bombardment, embryogenic cultures were placed on proliferation medium without any selective agent for 7 days. Selection of transformed material was done by transferring embryo cultures to proliferation medium augmented with 30 mg l^{-1} hygromycin, pH 5.8 (Sigma, St. Louis). Surviving dark green clumps of embryogenic cultures, after 5–6 months of selection on 30 mg l^{-1} hygromycin, were transferred onto maturation medium (MS-based medium containing 60 g l^{-1} maltose, 2 g l^{-1} phytagel, and 0.5 g l^{-1} activated charcoal, pH 5.8) for 21 days. Final maturation was achieved by transferring individual embryos onto maturation media and cultured until the embryos had grown to a length of 1.0 cm with visible cotyledons. Groups of six to eight embryos were desiccated in plastic Petri plates sealed with Parafilm and placed in a culture room for 3–7 days. Embryos were removed as they became creamy white in color and transferred to germination medium (MS-based medium containing 30 g l^{-1} sucrose and 2 g l^{-1} phytagel, pH 5.8). Most embryos began to form shoots and roots in 14–21 days, although some embryos required up to 8 weeks. These plantlets were transferred to Phytatrays (Sigma, St. Louis) containing half-strength B5 medium (Gamborg et al. 1968) for 14 days before being planted into artificial soil (ProMix BX, Premier Horticulture Inc., Red Hill, PA) and placed in humidity domes for acclimatization and subsequent transfer to the greenhouse.

Southern and northern blot analyses

Genomic DNA was isolated from transgenic and untransformed (Jack) soybean plants as per the manufacturer's instructions by homogenizing 100 mg of leaf tissue in 300 μl of Plant DNAzol® reagent (Invitrogen™, Life technologies, Carlsbad, CA). In order to eliminate RNA contamination in samples, 100 μg of RNase per ml of Plant DNazol was added at the beginning of the isolation procedure. Ten micrograms of total genomic DNA from each plant was digested overnight with *Hin*dIII; separated on a 0.7% agarose gel; and blotted onto a Zeta-probe® GT blotting membrane (Bio-Rad Laboratories, Hercules, CA). Hybridization was done overnight at 65°C in a phosphate-buffered solution (0.12 M sodium phosphate buffer, pH 7.2; 0.25 M sodium chloride; 1 mM EDTA; 7% sodium dodecyl sulfate) with a full-length GUS or γ-TMT gene random primed with α^{32}P dCTP using the Prime-It® II Random Primer Labeling Kit (Stratagene®, Cedar Creek, TX). After completion of the hybridization, the membrane was rinsed briefly with 2 × SSC and then washed successively by vigorous agitation at room temperature for 15 min in 2 × SSC and 0.1% SDS followed by 15 min in 0.5 × SSC and 0.1% SDS. Finally, the membrane was given a high stringency wash with 0.1 × SSC and 0.1% SDS at 65°C and exposed in a phosphorimager cassette (Molecular Dynamics, Sunnyvale, CA). The intensities of the bands were detected using the ImageQuanta™ software (Molecular Dynamics, Sunnyvale, CA).

Total RNA was isolated from 100 mg of fully expanded leaves, immature cotyledons and mature cotyledons collected from the greenhouse-grown transgenic and untransformed soybean plants using 1 ml of TRIzol® reagent (Invitrogen, Life technologies, Carlsbad, CA). Ten micrograms of total RNA was separated on 1.2% agarose gels containing formaldehyde and transferred onto a Zeta probe membrane. Hybridization and washings were done as described above for Southern blot hybridization.

HPLC analysis

Immature cotyledons collected from T0 plant and seed collected from T0 and T1 transgenic soybean plants were extracted and analyzed for tocopherol content with a method adapted from Savidge et al. (2002). Approximately 500 mg of tissue was ground in liquid nitrogen, and the resultant powder was transferred to 2 ml of 1% (w/v) pyrogallol (Sigma, St. Louis) in ethanol. The extract was vortexed and centrifuged at 7500 rpm for 2 min to remove large chunks of debris. The supernatant collected was filtered through a 0.2 μm filter (PTFE 13 mm syringe filters, Alltech, Deerfield, IL) and the filtrate was collected into a 4 ml glass vial with a Teflon-lined cap. Extracts were stored at -20°C until analyzed. A 0.75 ml aliquot of filtrate was then dried under nitrogen gas, and dried samples were resuspended in 0.75 ml of isooctane-tetrahydrofuran (THF) (85:15 v/v). Extracts (15-μl injections) were separated on a Dionex Summit HPLC system consisting of a P680 pump, an ASI-100 autosampler, a TCC-100 column oven at 24°C, and an RF-2000 fluorescence detector. The HPLC method followed the procedure of Darnoko et al. (2000), except that the mobile phase (isooctane-THF 97.5:2.5 v/v) was pumped onto a Phenomenex Luna silica column with a silica guard column, and the flow rate was 1.0 ml min^{-1}. Also, instead of UV detection, fluorescence detection was used, with excitation at 295 nm and emission at 330 nm (Goffman and Böhme 2001) and with detector sensitivity increased 32-fold above baseline. The α-, β-, γ- and δ-tocopherol standards, obtained from Sigma were dissolved and serially diluted in isooctane-THF (85:15 v/v). Standard concentrations ranged from 5 to 100 μM, and 10-μl volumes (5–1000 pmol) were injected. Linear calibration curves were generated for each standard with Chromeleon software (version 6.7) and used to determine tocopherol concentration and composition in seed extracts.

Lipid peroxidation

Lipid peroxidation in immature cotyledons, dry seed and 3-day-old germinating seed collected from T1 transgenic soybean plants was determined by estimating the malondialdehyde content (Heath and Packer 1968; Madhava Rao and Sresty 2000). The assay is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA); forming a MDA–TBA_2 adduct that absorbs light strongly at 532 nm. Approximately 100 mg of tissue was macerated in 2 ml of 0.1% trichloroacetic acid. The homogenate was centrifuged at 10,400 rpm for 5 min. For every 200 μl of the aliquot of the supernatant, 800 μl of 20% TCA containing 0.5% TBA was added. The mixture was heated at 95°C for 30 min and then cooled quickly in an ice bath. The resulting mixture was centrifuged at 10,400 rpm for 15 min and the absorbance of the supernatant was measured at 532 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The concentration of malondialdehyde content was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Results

The *Perilla frutescens* γ-TMT (PfTMT) protein shares a high degree of amino acid sequence similarity with *Glycine max* γ-TMT (68.3%) and Arabidopsis γ-TMT (67.4%) protein sequences and it showed a 49.1% similarity with the *Synechocystis* γ-TMT protein sequence (Fig. 2). The PfTMT protein-coding region has features that are consistent with its identity as an *S*-adenosylmethionine-dependent methyltransferase and a predicted 70 amino acid chloroplast targeting sequence (ChloroP 1.1 software; Emanuelsson et al. 1999). Two regions of sequence similarities called motifs 1 and 2 are seen in a broad spectrum of γ-tocopherol methyltransferases (Kagan and Clarke 1994). Both motifs 1 and 2 consist of a nine-residue consensus sequence: (N/K/S)(I/V)(V/L)D(V/L)GCGI and (V/A/W)RV(A/L) (A/K)PG(G/A)(R/T), respectively (Fig. 2).

Analyses of transgenic plants

Twenty-four putative transgenic soybean plants derived from six independent transformation experiments using the p1304*PfTMT* construct were tested for the presence of the transgene (Fig. 1). Southern blot analysis was carried out to verify the transgene integration pattern. Total genomic DNA was isolated from the putative transgenic lines, and the Southern blot hybridization of the *Hin*dIII digested DNA with the GUS gene probe indicated that one (T134) of the putative lines tested was positive (Fig. 3a). Results of the Southern blot hybridization analysis shown in Fig. 3a revealed the presence of three copies of the GUS gene in transgenic line T134. Further analysis of the transgenic line, T134, was confined to subsequent generations.

The expression pattern of the *γ-TMT* gene in the Southern positive line (T134) was verified by northern blot hybridization of total RNA isolated from different tissues, leaves, immature cotyledons and mature cotyledons. Northern blot analysis confirmed seed-specific expression of the *γ-TMT* gene (Fig. 3b). The level of *γ-TMT* gene expression is very prominent in immature cotyledons (5–7 mm) compared to the expression levels in the mature cotyledons (8–10 mm), and the expression levels were correlated with the total RNA levels shown in Fig. 3b.

Seed collected from T0 transgenic soybean plant (T134) were germinated and T1 seedlings were tested for GUS gene

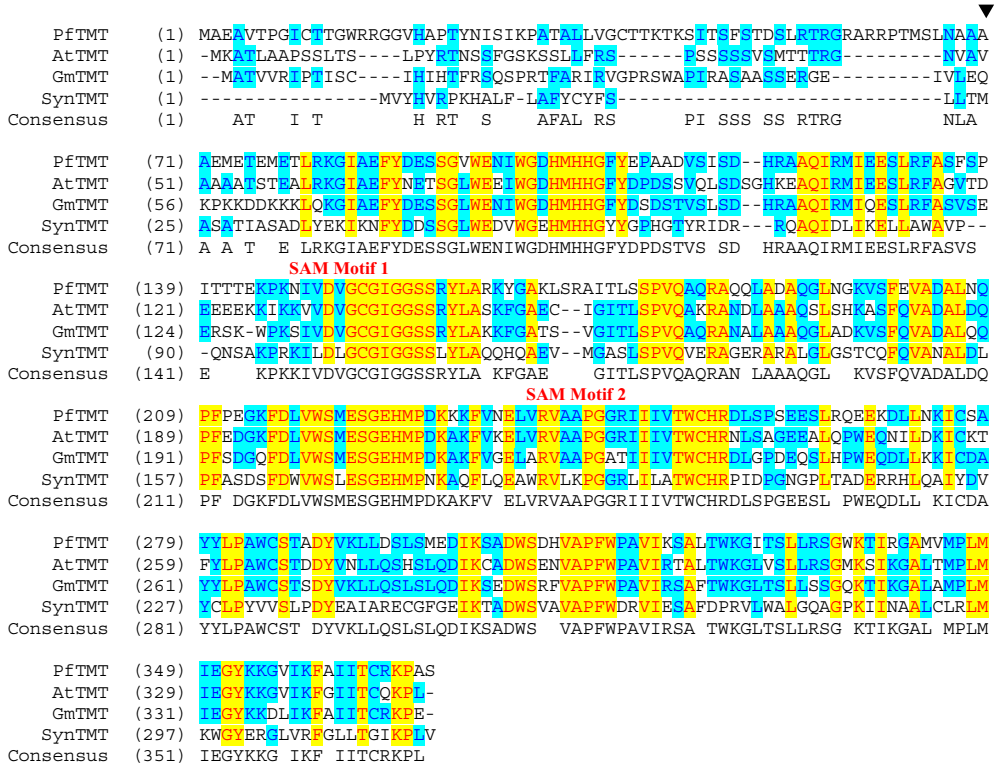

Fig. 2 Alignment of γ-TMT protein sequences from *Perilla frutescence* and three other organisms. The protein sequence of *Perilla frutescence* γ-TMT (PfTMT; GB#AF213481) was aligned with the *Arabidopsis thaliana* γ-TMT (AtTMT; GB#NM_105171), *Glycine max* γ-TMT (GmTMT; GB# AY960126) and *Synechocystis* PCC 6803 γ-TMT (SynTMT; GB# BA000022) protein sequences. Two motifs (SAM motifs 1 and 2) corresponding to conserved *S*-adenosylmethionine (SAM) binding domains (Kagan and Clarke 1994) are indicated. The inverted solid triangle (▼) indicates the predicted chloroplast transit peptide processing site in the PfTMT protein sequence (ChloroP 1.1 software; Emanuelsson et al. 1999)

expression by a histochemical assay. Out of 16 seedlings analyzed, 10 were GUS positive, and the leaf discs were very dark blue in color (data not shown). Southern blot analysis was carried out on the total genomic DNA isolated from leaf tissues of 10 T1 transgenic lines that were positive for the GUS histochemical test, using the full-length *γ-TMT* gene as a probe and revealed that all 10 lines also inherited the *γ-TMT* gene (Fig. 4a). All three copies of the inserts were found in T1 plants when probed with the GUS gene as was seen in Fig. 3a (data not shown), whereas hybridization with *γ-TMT* gene resulted in the presence of a single copy (Fig. 4a). This is because of the restriction enzyme (*Hin*dIII) selected to digest the genomic DNA. The p1304*PfTMT* vector has two *Hin*dIII restriction sites, one at the 5′ end of the *γ-TMT* gene and the other restriction site located at the 3′ end of OCS T sequence (Fig. 1). Therefore, restriction of soybean genomic DNA with *Hin*dIII enzyme excises the *γ-TMT* gene and OCS T cassette out and results in the appearance of a single band in the Southern blot (Fig. 4a). Northern blot hybridization of total RNA isolated from immature cotyledons collected from 10 different T1 lines confirmed the expression of the *γ-TMT* gene in all the lines tested (Fig. 4b). However, expression levels in T1 lines 1, 4, 8, and 10 were relatively low compared to other lines (Fig. 4b).

Analysis of tocopherol content

HPLC analysis performed on the alcohol extracts prepared from the green immature cotyledons and dry seed collected from T0 transgenic plant (T134) showed that the seed-specific overexpression of the *PfTMT* gene increased the α-tocopherol levels compared to the wild-type controls (Fig. 5b and c). In wild-type controls, the α- and γ-tocopherol contents in the immature cotyledons were 3.3 and 60.6 pmol mg^{-1} tissue, respectively, and in the seed the α- and γ-tocopherols were 43.9 and 361.5 pmol mg^{-1} tissue, respectively. In the case of transgenic line T134, the α- and γ-tocopherol contents in the immature cotyledons were 39.6 and 10.4 pmol mg^{-1} tissue, respectively (Fig. 5b). The α-tocopherol content in the seed collected from T134 transgenic line was 353.1 pmol mg^{-1} tissue, and γ-tocopherol was not detectable (Fig. 5c and Table 1). The β-tocopherol content in the immature cotyledons collected from the wild-type plants was almost zero, whereas the β-tocopherol content

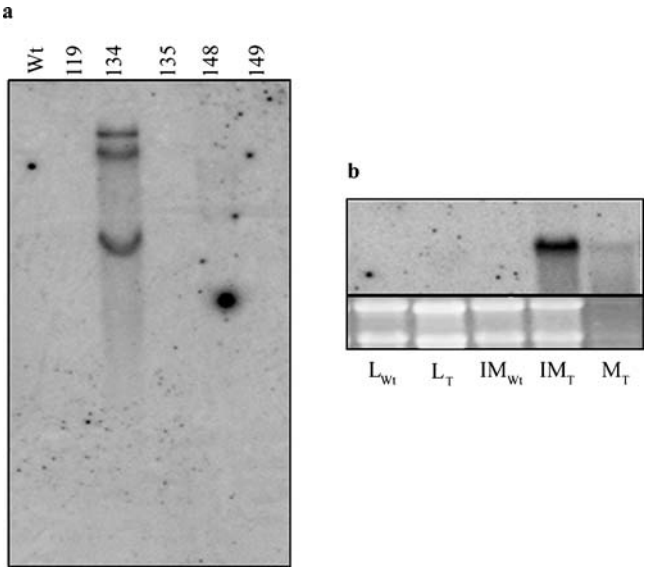

Fig. 3 Southern and northern blot analyses of transgenic soybean plants transformed with the p1304*PfTMT* construct. **a** Southern blot analysis of *Hin*dIII restricted total genomic DNA isolated from the putative transgenic soybean plants and hybridized with a full-length GUS gene probe. Wt: DNA isolated from the leaves of wild-type "Jack" soybean; Lanes 119–149 represent the DNA samples isolated from the leaves of putative transgenic plants. **b** Northern blot analysis of the total RNA isolated from different tissues of transgenic soybean plant (T134) and probed with full-length *γ-TMT* gene. L_{Wt}: RNA isolated from the leaves of wild-type "Jack" soybean; L_T: RNA isolated from the leaves of transgenic soybean plant (T134); IM_{Wt}: RNA isolated from the immature cotyledons of wild-type "Jack" soybean; IM_T and M_T: RNA isolated from the immature (IM) and mature (M) cotyledons collected from the transgenic soybean plant (T134)

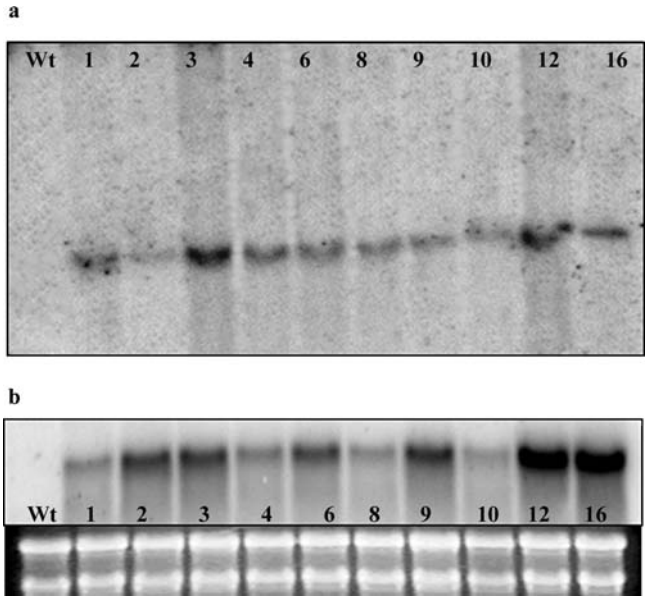

Fig. 4 Southern and northern blot analyses of T1 progeny of transgenic soybean line T134. **a** Southern blot analysis of *Hin*dIII restricted genomic DNA isolated from 10 different T1 lines and hybridized with *γ-TMT* gene probe. **b** Northern blot analysis on the total RNA isolated from the immature cotyledons collected from 10 T1 lines and probed with *γ-TMT* gene probe. Wt: wild-type "Jack" soybean; 1, 2, 3, 4, 6, 8, 9, 10, 12 and 16 represent T1 progeny of the transgenic soybean line T134

in immature cotyledons of the T134 transgenic line was 3.9 pmol mg^{-1} tissue. Conversely, the δ-tocopherol content in immature cotyledons was 6.2 and 1.9 pmol mg^{-1} tissue in the wild-type and transgenic lines, respectively (Fig. 5b). The β-tocopherol content increased from 4.3 in wild-type control seed to 79.2 pmol mg^{-1} tissue in transgenic seed (Table 1). Seed collected from the T0 transgenic line overexpressing the *γ-TMT* gene contained 81.7% of their total tocopherol pool as α-tocopherol and in the seed collected from the T1 transgenic lines the α-tocopherol content was 85–89% of the total tocopherol pool (Table 1). In addition, results obtained on the tocopherol content indicate that the δ-tocopherol pool in wild-type seed was converted into β-tocopherol in transgenic lines except in the seed collected from T134-3 T1 line where the δ-tocopherol levels were about one-thirtieth of the concentration found in wild-type seed (Table 1). On average, a 10.4-fold increase in the α-tocopherol content and a 14.9-fold increase in the β-tocopherol content was observed in T2 seed collected from T1 transgenic lines compared to wild-type controls (Table 1).

Table 1 Summary of the HPLC data on tocopherol contents in the seed collected from T0 and T1 transgenic soybean plants

	α-Tocopherol (pmol mg^{-1} tissue)	β-Tocopherol (pmol mg^{-1} tissue)	γ-Tocopherol (pmol mg^{-1} tissue)	δ-Tocopherol (pmol mg^{-1} tissue)	α-Tocopherol (%)	β-Tocopherol (%)
Wt	43.9 ± 4.2	4.3 ± 0.53	361.5 ± 15.5	112.8 ± 4.7	8.4	0.8
T134	353.1 ± 15.6	79.2 ± 5.4	–	–	81.7	18.3
T134-1	373.7 ± 27.1	62.1 ± 8.7	0.6 ± 0.0	–	85.6	14.2
T134-2	386.4 ± 24.7	53.5 ± 1.1	–	–	87.8	12.1
T134-3	353.0 ± 31.7	58.0 ± 5.0	6.1 ± 3.2	3.7 ± 0.0	83.9	13.8
T134-6	450.1 ± 24.3	55.7 ± 5.4	–	–	89.0	11.0
T134-9	375.9 ± 10.6	42.4 ± 6.1	–	–	89.9	10.1
T134-12	390.5 ± 24.0	51.4 ± 2.2	1.4 ± 0.7	–	88.1	11.6
T134-16	373.9 ± 16.0	53.3 ± 3.7	1.9 ± 0.5	–	88.3	12.4

Data represent the average of three replicates ± SD.

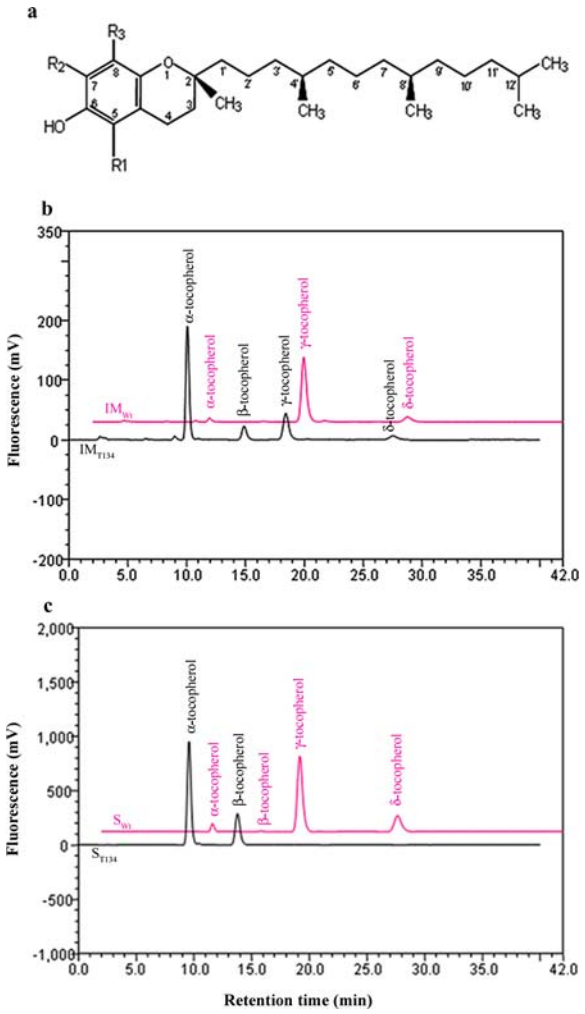

Fig. 5 Chemical structure of tocopherol and HPLC profiles of tocopherol contents in immature cotyledons and seed collected from transgenic soybean line (T134). **a** Basic structure of tocopherol showing the position of methyl groups (R_1, R_2 and R_3) on chromanol ring. The biological activities of α-(R_1=R_2=R_3=CH_3), β-(R_1=R_3=CH_3, R_2=H), γ-(R_1=H, R_2=R_3=CH_3) and δ-(R_1=R_2=H, R_3=CH_3) tocopherols vary with the number and position of methyl groups. **b** Overlay of HPLC profiles obtained for the alcohol extracts prepared from the immature cotyledons of wild-type "Jack" soybean (IM_{Wt}) and transgenic line (IM_{T134}) chromatographs. **c** Overlay of HPLC profiles obtained for the alcohol extracts prepared from the seeds of wild-type control (S_{Wt}) and transgenic line (S_{T134}) chromatographs

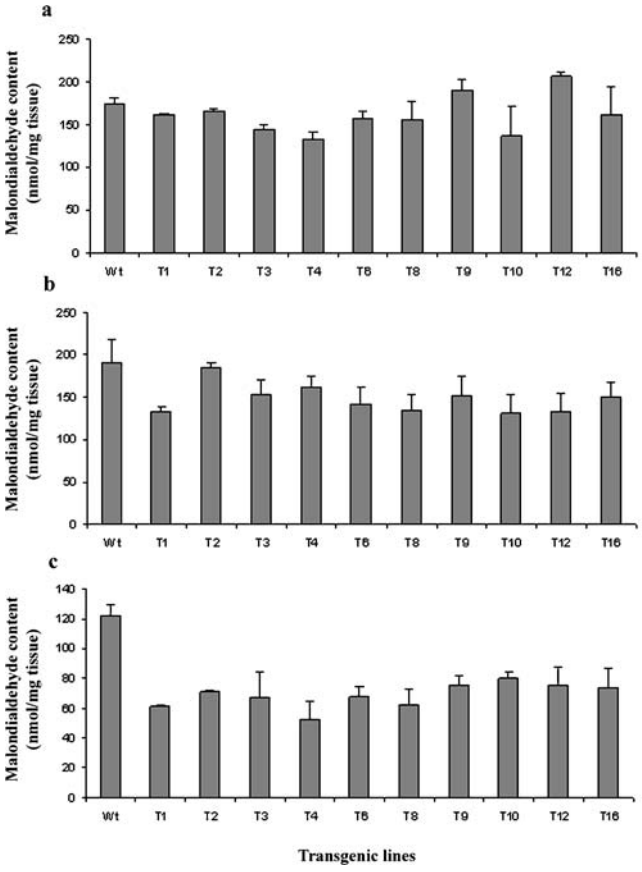

Fig. 6 Lipid peroxidation in T2 transgenic soybean lines. Lipid peroxidation was determined by estimating the malondialdehyde content in the immature cotyledons **a**, seed **b**, and 3 days after germinating seed **c**. Data represent the average of four replicates ± SD

Lipid peroxidation

The formation of malondialdehyde is a measure of lipid peroxidation. Therefore, to verify the role of α-tocopherol in protecting polyunsaturated fatty acids (PUFAs) from lipid peroxidation, the levels of lipid peroxy radicals were determined by measuring the malondialdehyde content in the immature cotyledons, dry seed and 3-day-old germinating seed collected from T1 transgenic plants. No significant differences were observed in the lipid peroxidation products measured in immature cotyledons collected from transgenic lines and wild-type controls (Fig. 6a). On average there was a 21.8% decrease in lipid peroxidation products in the seed collected from 10 T1 transgenic lines compared to wild-type controls (Fig. 6b). Interestingly, we found an average of 44% reduction in lipid peroxidation products in the germinating seed of transgenic lines compared to wild-type controls (Fig. 6c). Irrespective of the levels of lipid peroxidation products, transgenic plants showed no specific differences from wild-type controls in flowering, seed development, seed germination, and seedling growth (data not shown).

Discussion

Tocopherols are a group of naturally occurring lipophilic compounds with antioxidant activities, produced only in photosynthetic organisms (Sheppard et al. 1993). Among the four different tocopherols, γ-tocopherol is frequently the predominant form of vitamin E found in plant seed, and in products such as vegetable oils. However, based on

fetal resorption assays, α-tocopherol was found to contain the highest vitamin E activity (Bunyan et al. 1961; Weiser et al. 1986, 1996). Since soybean oil accounts for the major edible oil consumption of the population, there has been an interest in recent years to generate transgenic soybean plants for the increased α-tocopherol content by overexpressing the *γ-TMT* gene (Kim et al. 2005; Van Eenennaam et al. 2003).

In this study we have shown that the seed-specific overexpression of the *P. frutescens γ-tocopherol methyltransferase* (*PfTMT*) gene increased the α-tocopherol content from 8.41% of total tocopherol content in wild-type seed to 81.67% of total tocopherol in the T1 transgenic soybean seed (Table 1). In T2 transgenic soybean seed, the average α-tocopherol content was 87.51% of the total tocopherol pool, which corresponds to a 10.4-fold increase in α-tocopherol content compared to wild-type controls. In addition, β-tocopherol, which was less than 1% of the tocopherol content of wild-type seed, increased to 18.33% of total tocopherol content in the T1 transgenic seed and 12.19% of total tocopherol content in the T2 seed (Table 1). This indicates that the overexpression of PfTMT, leading to methylation at the C-5 site of both γ- and δ-tocopherols, resulted in nearly complete conversion of all tocopherols present in T1 and T2 soybean seed to α- and β-tocopherols (Fig. 5a and c, and Table 1). Given the differing vitamin E potencies of α-, β-, γ- and δ-tocopherols (100, 50, 10 and 3% relative vitamin E activity, respectively) (Kamal-Eldin and Appelqvist 1996; Sheppard et al. 1993), targeted overexpression of the *PfTMT* gene was calculated to have increased the vitamin E activity of T2 transgenic soybean seed 4.8-fold compared to the vitamin E activity of wild-type seed.

Similar attempts to increase the α-tocopherol content in soybean seed have been done using the *Arabidopsis thaliana γ-TMT* (*At-VTE4*) gene. Kim et al. (2005) reported that the overexpression of *At-VTE4* gene in soybean seed resulted in a fourfold increase in the α-tocopherol content compared to non-transgenic plants. In another study, Van Eenennaam et al. (2003) reported that the overexpression of the *At-VTE4* gene in soybean seed converted γ-tocopherol almost completely into α-tocopherol, a 7-fold increase to 75% of total tocopherol content, and δ-tocopherol almost completely into β-tocopherol, a 10-fold increase to 25% of total tocopherol content. Van Eenennaam et al. (2003) have further shown that overexpression of *At-VTE4* together with *At-VTE3* (2-methyl-6-phytylbenzoquinol methyltransferase) resulted in an eightfold increase in α-tocopherol content and up to a fivefold increase in the soybean seed vitamin E activity. In this study, we were able to show a substantial increase in the α-tocopherol content of soybean seed by overexpressing the *PfTMT* gene alone. Presently we do not know if the high level of α-tocopherol in the transgenic soybean line is due to a higher activity of the PfTMT protein or specific for this particular integration event.

Lipid peroxidation has been established as a major mechanism of cellular injury in many biological systems (Munne-Bosch and Alegre 2002). Polyunsaturated lipids are especially susceptible to this type of damage when in an oxidizing environment, and they can react to form lipid peroxides. Seed germination and seedling development are an oxidative bottleneck for plants in which tocopherols play an essential role (Sattler et al. 2004). Therefore, lipid peroxidation products were measured in immature cotyledons, seed and 3-day-old germinating seed collected from T1 transgenic soybean lines. Lipid peroxidation products measured in immature cotyledons did not show any significant differences compared to wild-type controls (Fig. 6a). However, decreases in lipid peroxidation products were observed in dry seed and 3-day-old germinating seed of transgenic soybean lines compared to wild-type controls (Fig. 6b and c).

The principal role of α-tocopherol as an antioxidant is believed to be in scavenging lipid peroxy radicals, which are the chain-carrying species responsible for propagating lipid peroxidation (Burton and Ingold 1986; Liebler 1993). Indirect evidence suggests that tocopherols perform antioxidant and radical quenching functions in plants similar to those in animals (Fryer 1992). Results presented here on lipid peroxidation suggests that α-tocopherol probably has a role in preventing oxidative damage to lipid components during seed storage and seed germination (Fig. 6).

Many studies have demonstrated that the antioxidant activity of tocopherol molecules has the potential to positively impact human and animal health (Bramley et al. 2000; Fryer 1992; Fukuzawa and Gebicki 1983; Neely et al. 1988). In the present study, by overexpressing the *P. frutescens γ-TMT* gene, we were able to improve the tocopherol composition (10.4-fold increase in α-tocopherol content), which is expected to increase vitamin E activity by 4.8-fold in the soybean seed. In conclusion, overexpression of a *PfTMT* gene resulted in nearly complete conversion of all tocopherols present in the soybean seed to α- and β-tocopherols and the improvement in the α-tocopherol content and eventually the improvement in the vitamin E activity of the transgenic soybean seed. These results demonstrate that through the use of transgenic technology, there is a potential to significantly increase the dietary vitamin E intake.

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